

Does T helper differentiation correlate with resistance or susceptibility to infection with *L. major*? Some insights from the murine model

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The murine model of *Leishmania major* infection has been an invaluable tool in understanding T helper differentiation *in vivo*. The initial evidence for a role of distinct CD4⁺ T helper subsets in the outcome of infection was first obtained with this experimental model. The development of CD4⁺ Th1 cells was associated with resolution of the lesion, control of parasite replication, and resistance to re-infection in most of the mouse strains investigated (i.e., C57BL/6). In contrast, differentiation of CD4⁺ Th2 cells correlated with the development of unhealing lesions, and failure to control parasite load in a few strains (i.e., BALB/c). Since these first reports, an incredible amount of effort has been devoted to understanding the various parameters involved in the differentiation of these, and more recently discovered T helper subsets such as Th17 and T regulatory cells. The discovery of cross-talk between T helper subsets, as well as their plasticity force us to reevaluate the events driving a protective/deleterious T helper immune response following infection with *L. major* in mice. In this review, we describe the individual contributions of each of these CD4⁺ T helper subsets following *L. major* inoculation, emphasizing recent advances in the field, such as the impact of different substrains of *L. major* on the pathogenesis of disease.

Keywords: *Leishmania*, *L. major*, T helper cells, Th1, Th2, Th17, T_{reg}, IL-17

INTRODUCTION

The first description of the existence of two subsets of CD4⁺ T cells secreting distinct cytokines was made already 25 years ago (Mosmann et al., 1986). Two CD4⁺ T helper subsets named Th1 and Th2, first described *in vitro*, were shown to produce distinct cytokines with Th1 cells secreting IFN γ , and Th2 cells secreting IL-4, IL-13, and IL-5. The first evidence for a functional role of these CD4⁺ T helper subsets *in vivo* was obtained with the experimental model of cutaneous leishmaniasis, where *Leishmania major* promastigotes were inoculated subcutaneously in mice (Scott et al., 1988; Heinzel et al., 1989). Initially, resistance and susceptibility were shown to be correlated with the development of a CD4⁺ Th1 or Th2 response, respectively. Recently, several other T helper subsets have been identified first *in vitro*, and then *in vivo*. Among these, Th17 as well as the T regulatory (T_{reg}) cells were shown to influence the pathogenesis of disease in *L. major*-infected mice.

Among the factors driving the differentiation of specific T helper subsets, cytokines present at the site of parasite inoculation and in the draining lymph node (dLN) were shown to be critical. In addition, the dose of antigen, the strength of T cell receptor signaling, and the recognition of pathogen-associated molecular patterns were also shown to influence the differentiation of T helper subsets in several experimental models of infection. Furthermore, the plasticity of some of the CD4⁺ T helper subsets, reviewed in O'Shea and Paul (2010) complicates our understanding of the

factors involved in susceptibility or resistance to infection. Following inoculation of *L. major*, other parameters such as the size of the parasite inoculum, the mode (needle versus sand fly), and site of parasite inoculation, as well as the substrain of *L. major* further influence T helper differentiation and the outcome of the disease in this experimental model.

In this review we will focus on experimental leishmaniasis caused by *L. major*, one of the most extensively studied models of cutaneous leishmaniasis. We will (1) review experimental evidence revealing that the Th1/Th2 paradigm and its relevance to susceptibility or resistance to infection with *L. major* is not as strict as previously thought, and (2) discuss the impact that different *L. major* substrains (Table 1) have on the differentiation of T helper cells and susceptibility to infection.

THE FACTORS INVOLVED IN THE DIFFERENTIATION OF Th1 CELLS AND CONTROL OF LESION DEVELOPMENT FOLLOWING *L. MAJOR* INOCULATION

Several parameters are involved in the differentiation of naïve CD4⁺ T cells into CD4⁺ Th1 cells. Most laboratory mouse strains (C3H/He, CBA, C57BL/6, 129Sv/Ev) infected with *L. major* develop a small lesion that heals spontaneously, and these mice are resistant to re-infection. The healing phenotype has been correlated with the development of a CD4⁺ Th1 response, characterized by the generation of CD4⁺ T cells secreting IFN γ , a critical

Table 1 | Origins of the *L. major* substrains discussed in this review.

| <i>L. major</i> substrain | WHO code name | Origin of isolate | Country |
|---------------------------|---------------------|-------------------|---------|
| LV39 | MRHO/SU/59/P | Gerbil | Russia |
| Friedlin | MHOM/IL/80/Friedlin | Human | Israel |
| IR173 | MHOM/IR-173 | Human | Iran |
| FEBNI | MHOM/IL/81/FEBNI | Human | Israel |
| Sd ^a | MHOM/SN/74/Seidman | Human | Senegal |

^aIsolated from a patient with a chronic lesion refractory to chemotherapy.

cytokine triggering macrophage activation that leads to parasite killing.

The critical role of IFN γ in the control of the infection was first determined in mice treated with anti-IFN γ antibodies, and later using mice deficient in IFN γ . These mice were unable to control lesion size and parasite growth, developing a CD4⁺ Th2 response (Belosevic et al., 1989; Scott, 1991). Mice lacking the IFN γ receptor also failed to control lesion size and parasite growth confirming that IFN γ signaling is required in the healing process (Wang et al., 1994; Swihart et al., 1995). However, despite the development of unhealing lesions, C57BL/6 IFN γ R^{-/-} mice infected with the *L. major* LV39 substrain developed a CD4⁺ Th1 response (Swihart et al., 1995), while 129/Sv/Ev IFN γ R^{-/-} mice infected with the *L. major* IR173 substrain developed a Th2 immune response. Different substrains of *L. major* have been shown to induce distinct immune responses (Table 2), suggesting that the differences measured in these two studies initially attributed to the different genetic backgrounds of the infected mice, may result from the use of distinct *L. major* substrains. Further support in the importance of IFN γ in the control of *L. major* infection, was demonstrated using mice deficient in T-bet, the master transcription factor of Th1 cells, that also renders otherwise resistant mouse strains susceptible to infection (Szabo et al., 2002).

Following infection with *L. major*, IFN γ is mainly secreted by CD4⁺ Th1 cells and NK cells. The role of NK cell-derived IFN γ in CD4⁺ Th1 differentiation following *L. major* infection is currently controversial and may also depend on the *L. major* substrain. Neutralization of IL-12 in C57BL/6 mice prevents the early production of IFN γ by NK cells, abrogating the development of Th1 cells and leads to susceptibility to *L. major* Friedlin, Sd, and FEBNI strains (Laskay et al., 1993; Sypek et al., 1993; Scharton-Kersten and Scott, 1995). However, mice deficient in both T cells and IFN γ reconstituted with wild type (WT) CD4⁺ T cells as the sole source of IFN γ could differentiate functional Th1 cells upon challenge with *L. major* IR173 (Wakil et al., 1998), demonstrating that the IFN γ derived from NK cells is dispensable for Th1 differentiation using this *L. major* substrain. Furthermore, NK cells appeared to be dispensable for the development of CD4⁺ Th1 cells following infection with *L. major* LV39 substrain (Satoskar et al., 1999). It remains to be investigated if different substrains of *L. major* parasites induce distinct NK cell functions.

Th1 development subsequent to *L. major* infection has been associated with lesion healing. The IFN γ secreted by CD4⁺ Th1 cells synergizes with TNF to activate macrophage leishmanicidal

activity. In absence of TNF or TNFR, *L. major*-infected mice on the C57BL/6 genetic background develop functional CD4⁺ Th1 cells, are able control parasite load with some variations depending on the *L. major* substrain inoculated (Vieira et al., 1996; Nashleanas et al., 1998; Kanaly et al., 1999; Wilhelm et al., 2001; Chakour et al., 2003; Ritter et al., 2004). However, these mice were unable to heal their lesions, revealing that TNF, and particularly the trans-membrane form of TNF (Allenbach et al., 2008) is critical in the resolution of the inflammatory lesion, a process distinct from the development of CD4⁺ Th1 cells.

Lack of correlation between Th1 differentiation and control of lesion size in C57BL/6 mice was also observed following inoculation of the *L. major* Seidman (Sd) strain. C57BL/6 mice inoculated with the Sd strain developed unhealing lesions despite the development of a strong Th1 immune response (Anderson et al., 2005). In this case, a population of Th1 cells secreting both IFN γ and IL-10, as well as regulatory T cells were later shown to prevent the healing potential in this infection (Anderson et al., 2009). Collectively, these experiments reveal that the control of the inflammatory lesion involves several factors not always linked to the development of CD4⁺ Th1 cells.

IL-12 is a key cytokine leading to the generation of a protective CD4⁺ Th1 immune response following *L. major* infection. IL-12p70 is a heterodimeric molecule primarily secreted by antigen presenting cells (APCs), formed by the IL-12p40 and IL-12p35 subunits (Trinchieri et al., 2003). Mice deficient in either IL-12p40 or IL-12p35 are unable to control infection (Mattner et al., 1996, 1997; Park et al., 2002) and mice deficient in the IL-12 signaling molecule STAT4 developed non-healing lesions following *L. major* infection (Jacobson et al., 1995; Stamm et al., 1999). Conversely, susceptible BALB/c mice treated with exogenous IL-12 developed a Th1 response leading to parasite control (Heinzel et al., 1993; Sypek et al., 1993; Sakai et al., 2000). IL-12 not only initiates the development of a Th1 response to *L. major* infection but it is also required to maintain Th1 cells during infection (Park et al., 2000, 2002; Stobie et al., 2000), and continued production of IL-12 during *L. major* infection is necessary for the development of IFN γ -producing Th1 effector cells from a plastic central memory CD4⁺ T cell pool (Pakpour et al., 2008).

In addition to IL-12, several other cytokines such as IL-18 and IL-27 can also contribute to CD4⁺ Th1 differentiation. IL-18, a member of the IL-1 cytokine family, is primarily produced by macrophages and dendritic cells (DCs) and serves as a cofactor during IL-12-mediated Th1 responses by optimizing IFN γ production from effector Th1 cells and activating NK cells. IL-12 upregulates the IL-18 receptor preferentially on Th1 cells suggesting a synergistic effect of IL-12 and IL-18 on the development of a Th1 response, reviewed in Nakanishi et al. (2001). Even though IL-18 is dispensable during *L. major* infection in C57BL/6 mice (Monteforte et al., 2000), the role of IL-18 in mediating protection appears to depend on the genetic background of the mouse strain (Xu et al., 2000; Wei et al., 2004). Administration of rIL-18 alone in BALB/c mice exacerbated the disease (Ohkusu et al., 2000; Li et al., 2004), revealing that IL-18 is not sufficient to induce Th1 differentiation in these Th2-prone mice. While IL-18 may not be required for the induction of a Th1 response after *L. major*

Table 2 | Differential immune responses to *Leishmania major* substrains.

| L. major substrain | Control of infection | IL-4 | IL-10 | IL-17 | References |
|--------------------|--------------------------|---|---|---|--|
| LV39 | C57BL/6 (R) ^a | Over-expression of IL-4 (S) | n.d. | IL-17 ^{-/-} (NA) | Leal et al. (1993), Noben-Trauth et al. (1996, 1999, 2003), Radwanska et al. (2007) |
| | BALB/c (S) ^b | IL-4 ^{-/-} and IL-4Rα ^{-/-} (S); CD4 ⁺ specific IL-4Rα ^{-/-} (R) | IL-10 ^{-/-} or IL-10R blockade (R) | IL-17 ^{-/-} (NA) | Tacchini-Cottier and Launois (unpublished data) |
| Friedlin | C57BL/6 (R) | Transient IL-4 in low dose inf but lesion resolution | IL-10 ^{-/-} or IL-10R blockade (R); prevent parasite elimination from skin | IL-17 ^{-/-} or rIL-17 (NA) | Belkaid et al. (2001), Kane and Mosser (2001), Belkaid et al. (2002), Noben-Trauth et al. (2003), Uzonna et al. (2004), Wu et al. (2010), Lopez Kostka et al. (2009) |
| | BALB/c (S) | IL-4Rα ^{-/-} (S) | IL-10 ^{-/-} (R) | IL-17 ^{-/-} partially control parasitemia but no lesion resolution | |
| IR173 | C57BL/6 (R) | rIL-4 injection (NA) | n.d. | n.d. | Sadick et al. (1991), Heinzel and Maier (1999), Noben-Trauth et al. (1999, 2003) |
| | BALB/c (S) | IL-4 ^{-/-} partially control inf but IL-4Rα ^{-/-} fully (R) | IL-10 ^{-/-} or IL-10R blockade partially (R) | n.d. | |
| FEBNI | C57BL/6 (R) | n.d. | n.d. | n.d. | Kopf et al. (1996), Kopf et al. (2003), Radwanska et al. (2007) |
| | BALB/c (S) | IL-4 ^{-/-} and IL-4Rα ^{-/-} partial control of inf but (S); CD4 ⁺ specific IL-4Rα ^{-/-} (R) | n.d. | n.d. | |
| Sd | C57BL/6 (S) | IL-4 ^{-/-} and IL-4R blocked (S); undetectable IL-4 in WT | IL-10R blockade (R); IL-10 production by T cells controls Th1 response and pathology; IL-10 associated with unhealing lesions | Weak IL-17 production modulated by IL-27 | Kopf et al. (1999, 2003), Sacks and Noben-Trauth (2002), Anderson et al. (2005, 2009) |
| | BALB/c (S) | IL-4 ^{-/-} (NA) | IL-10R blockade partially (R) | n.d. | |

^aR: mice develop self-healing lesion, control parasite load, and are resistant to re-infection; ^bS: susceptible, unhealing lesion, uncontrolled parasite load. NA: disease phenotype not altered; n.d., not determined.

inoculation, these data collectively suggest that this cytokine can increase IFN γ production and thus potentiate the effects of IL-12 in the development of a CD4⁺ Th1 response.

IL-27 is a cytokine closely related to IL-12 which is produced by phagocytes. It can also induce the differentiation of naïve T cells toward Th1 cells through the early induction of IFN γ production by T cells and NK cells (Pflanz et al., 2002). During *L. major* infection, C57BL/6 mice deficient in the IL-27R (WSX-1^{-/-}) showed early impaired IFN γ response and increased IL-4 secretion and were transiently more susceptible than WT controls (Yoshida et al., 2001); however, these mice eventually developed a *L. major*-specific robust IFN γ response and healed their lesions in the absence of IL-4 (Artis et al., 2004). Further studies confirmed a transient role for IL-27 in Th1 differentiation in C57BL/6 mice, which is probably linked to its control of early IL-4 secretion (Anderson et al., 2009). A role for IL-27 in the control of Th17 differentiation was also described, linking Th1 and Th17 differentiation (Anderson et al., 2009).

THE FACTORS INVOLVED IN THE DIFFERENTIATION OF Th2 CELLS AND SUSCEPTIBILITY FOLLOWING *L. MAJOR* INOCULATION IN BALB/c MICE

The murine model of infection with *L. major* provided the first association between expansion of CD4⁺ Th2 cells and progressive disease (Scott et al., 1988; Heinzel et al., 1989). Given that mRNA levels or the production of IFN γ were similar early in infection in resistant and susceptible mice (Morris et al., 1993), the strict correlation between susceptibility to infection and failure to mount a Th1 response was put into question. These experiments suggested that the development of pathology observed in BALB/c mice inoculated with *L. major* does not result from a defective Th1 development.

Thereafter, an important role for the IL-4 cytokine in mediating Th2 cell differentiation and susceptibility to infection with *L. major* was established. It was first observed that administration of neutralizing antibodies against IL-4 reversed the disease phenotype in BALB/c mice, rendering them resistant to infection (Sadick et al.,

1990). Conversely, IL-4 transgenic C57BL/6 mice expressing IL-4 failed to clear the infection (Leal et al., 1993). Furthermore, several studies established that the IL-4 produced during the early phases of infection is important for the development of CD4⁺ Th2 cells, but the cellular source of this early IL-4 production remains a matter of debate. CD4⁺ T cells with the V β 4V α 8 T cell receptor were shown to be the source of this early IL-4 secretion in response to the immunodominant epitope from *Leishmania* activated C kinase (LACK; Julia et al., 1996; Launois et al., 1997a,b, 2002; Himmelrich et al., 2000). However, subsequent studies questioned the role of LACK-specific CD4⁺ T cells as the sole source of IL-4 needed for the development of a Th2 response and thus the susceptibility of BALB/c mice. First, precursor frequency expansion and IL-4 mRNA expression of LACK-specific V β 4V α 8 CD4⁺ T cells were shown to be comparable in both *L. major* susceptible and resistant strains of mice (Scott et al., 1996; Stetson et al., 2002). In addition, whereas inoculation of LACK-mutated *L. major* parasites, that are not able to activate the V β 4V α 8 CD4⁺ T cells, resulted in reduced expansion of these cells *in vivo*, the frequency of IL-4-producing cells in infected mice was similar to those obtained following injection of WT *L. major* parasites (Kelly and Locksley, 2004). Collectively, these results demonstrated that the early IL-4 production by CD4⁺ T cells was important but not the only signal required for Th2 differentiation in BALB/c mice.

In parallel with these data, further experiments performed in IL-4 deficient mice strengthened the importance of other factors in driving Th2 responses during *L. major* infection. IL-4 deficient BALB/c mice infected with the *L. major* LV39 strain remained susceptible to infection (Noben-Trauth et al., 1996) while those infected with two other *L. major* substrains (FEBNI or IR173), were able to control the infection (Kopf et al., 1996; Noben-Trauth et al., 1999). Interestingly, these studies revealed not only that other factors than IL-4 contribute to susceptibility to *L. major*, but that different substrains of *L. major* can induce distinct immune responses in BALB/c mice. These data demonstrated that other Th2 cytokines such as IL-13 and IL-10 also contribute to susceptibility to infection.

A role for IL-13 in susceptibility to infection with *L. major* was demonstrated in IL-4R α deficient BALB/c mice. IL-4R α is a component of both the IL-4R and the IL-13R thus both IL-4 and IL-13 are inactive in these mice. Comparing the outcome of infection in these and IL-4^{-/-} mice allowed the determination of the role of IL-4 and IL-13 in the development of a Th2 response. Although IL-4^{-/-} BALB/c mice infected with *L. major* IR73 strain partially controlled the infection, IL-4R α ^{-/-} mice inoculated with the same strain of *L. major* were fully resistant (Noben-Trauth et al., 1999), demonstrating the importance of IL-13 in the susceptibility of mice infected with *L. major* IR173. These results were corroborated by other studies showing that IL-13 deficient BALB/c mice became resistant to infection and that over-expression of IL-13 in resistant C57BL/6 rendered these mice susceptible to infection with *L. major* LV39 (Matthews et al., 2000). However, since injection of IL-13R α 2 fusion protein, which blocks the biological activity of IL-13 in IL-4^{-/-} BALB/c mice infected with *L. major* LV39 did not modify the course of infection and parasite burden (Kropf et al., 1999) and since both IL-4^{-/-} and IL-4R α ^{-/-} BALB/c mice infected with *L. major* LV39 remained susceptible

to infection (Noben-Trauth et al., 1999), mechanisms/cytokines other than IL-4 and IL-13 are likely to be involved in the susceptibility to infection. In contrast, a protective role for an IL-4/IL-13 responsive non-T cell in the development of a healing phenotype following infection with *L. major* LV39 was reported in BALB/c mice with the abrogation of IL-4 and IL-13 signaling selectively in CD4⁺ T cells (Radwanska et al., 2007).

In addition to IL-4 and IL-13, a crucial role for IL-10 in susceptibility was shown in BALB/c mice doubly deficient for the IL-4R α and IL-10, as these mice were resistant to infection with *L. major* LV39 (Noben-Trauth et al., 2003). Interestingly, IL-10^{-/-} BALB/c mice were more resistant to infection with *L. major* Friedlin than WT mice (Kane and Mosser, 2001). The cellular source of IL-10 production is another factor influencing the effect of IL-10 on susceptibility to infection with *L. major*. C57BL/6 mice expressing an IL-10 transgene under the IL-2 promoter, directing IL-10 secretion in T cells, remained resistant to *L. major* (Hagenbaugh et al., 1997). In contrast, C57BL/6 mice that express an IL-10 transgene under the control of the MHC promoter, directing IL-10 expression mainly in APCs, were susceptible to infection with *L. major* (Groux et al., 1999). In this context, we have recently demonstrated that a subset of *L. major*-specific B cells expressing the CD1B and CD5 antigens are necessary for the development of a Th2 cell response in BALB/c mice infected with the *L. major* LV39 strain through the production of IL-10 (Ronet et al., 2010).

Altogether these studies showed that several cytokines are involved in the development and maintenance of susceptibility to infection in response to *L. major* in susceptible BALB/c mice, and that the requirement of IL-4 and/or IL-13 and/or IL-10 for the development of a Th2 response is linked with several factors including the particular substrain of *L. major* inoculated (Table 2). Since these three cytokines have powerful inhibiting effects on macrophage activation and on IFN γ -mediated parasite killing (Bogdan et al., 1996), a small production of any of these cytokines could be sufficient to have an impact on parasite control.

In this line, different substrains of *L. major* were shown to influence macrophage activation and the ability to kill *Leishmania* parasites. For instance, killing of *L. major* LV39 requires greater levels of IFN γ than killing of the IR173 substrain (Noben-Trauth et al., 2003). Thus, although resistance to infection with *L. major* IR173 can be induced by the removal of IL-4, rendering BALB/c mice resistant to infection with *L. major* LV39 requires the inactivation of not only IL-4 but also of IL-13 and IL-10 in order for IFN γ to reach the threshold activity needed for parasite killing.

Altogether, the murine model has been crucial in the understanding of the multiple factors involved in susceptibility to *L. major* infection. Numerous evidences point to IL-4 as a critical cytokine leading to development of susceptibility to *L. major* in BALB/c mice, however additional factors including IL-10 and IL-13 also contribute to the pathology of the disease. It is likely that other factors yet to be described, some of them upstream of cytokines, will likely contribute to susceptibility to infection.

THE DEVELOPMENT OF Th17 CELLS FOLLOWING *L. MAJOR* INOCULATION

Th17 cells are a recently described T helper cell subset producing primarily IL-17A, IL-17F, and IL-22 as well as many other

cytokines including TNF, IL-21, and GM-CSF. ROR γ t and ROR α are the two master transcription factors involved in Th17 differentiation, but other transcription factors including STAT3, IRF4, and BATF also contribute to this process (reviewed in Hirota et al., 2010). The development of Th17 cells in mice and then in humans was initially described as requiring TGF β and IL-6, and to be inhibited by IL-4 and IFN γ (reviewed in Korn et al., 2009). Recently Th17 cells were also shown to differentiate from naïve CD4 $^{+}$ T cells in absence of TGF β , when exposed to IL-6, IL-23, and IL-1 β (Ghoreschi et al., 2010). Subsequently, two distinct Th17 subsets were identified, conventional Th17 cells [obtained *in vitro* in response to TGF β and IL-6 (Th17 β)], and inflammatory Th17 cells, obtained in response to IL-6 and IL-23, Th17(23). Different function and transcriptional profiles were ascribed to these subsets, with Th17 β cells expressing more IL-9, IL-10, and CCL10, while Th17(23) cells express higher levels of Tbx21, IL33, and CXCR3 (Ghoreschi et al., 2010).

Leishmania major susceptible mouse strains such as BALB/c mice develop unhealing lesions that are characterized by the presence of parasites and a persistent neutrophil infiltration at the lesion site (Beil et al., 1992; Tacchini-Cottier et al., 2000; Ribeiro-Gomes et al., 2004). One of the hallmarks of Th17 cells is the induction of the secretion of chemokines attracting neutrophils by various cell types, so it was of interest to investigate if Th17 cells played a role in the persistence of neutrophils observed in lesions of mice susceptible to *L. major*. The group of von Stebut measured higher levels of IL-17 and IL-23 in dLNs of BALB/c mice than in C57BL/6 mice inoculated with the *L. major* Friedlin substrain, suggesting the presence of inflammatory Th17 cells in dLN of BALB/c mice. DC-derived IL-23 was thought to induce and maintain Th17 cells in these mice. In addition to Th17-derived IL-17, secretion of IL-17 by recruited neutrophils appeared to also be important in mediating the persistence of an inflammatory lesion. IL-17A $^{-/-}$ BALB/c mice inoculated with a high dose (2×10^5) or low dose (10^3) of *L. major* Friedlin developed smaller lesion volume and parasite load (Lopez Kostka et al., 2009). However, the Th2 profile measured in dLNs of *L. major*-infected IL-17A $^{-/-}$ mice was similar to that of control mice, once more separating the Th2 response from the development of unhealing lesions. Of interest, we did not see any difference in lesion size or parasite control when IL-17 $^{-/-}$ BALB/c mice were inoculated with a high dose of *L. major* LV39 (Launois and Tacchini-Cottier, unpublished data); in addition, IL-23 $^{-/-}$ mice on a C57BL/6 genetic background infected with *L. major* LV39 did not show any difference in the control of inflammatory lesions nor the differentiation of Th1 cells (Launois and Tacchini-Cottier, unpublished data). Collectively, these data reveal that different substrains of *L. major* may also impact the levels of IL-17 and/or Th17 cells in susceptibility to infection.

Interestingly, neutrophil recruitment was different in *L. major*-infected IL-17 $^{-/-}$ and control mice from 2 to 3 weeks post parasite inoculation, but did not differ significantly during the first week of infection (Lopez Kostka et al., 2009). IL-17 contributes to the early recruitment of neutrophils after *L. major* LV39 inoculation (Tacchini-Cottier and Launois, unpublished data), suggesting that compensatory mechanisms may induce early neutrophil recruitment in IL-17 $^{-/-}$ mice. These data support the current hypothesis that the recruitment of neutrophils during the first days following

L. major inoculation involves multiple factors including CXCL6 (GCP-2; Uyttenhove et al., 2011), and possibly CXCL1 (KC), CXCL2 (MIP-2), as well as parasite-derived factors, reviewed in Charmoy et al. (2010).

A detrimental role for Th17 cells on lesion development was also revealed following inoculation of the *L. major* Sd substrain which causes non-healing lesions in otherwise *L. major* resistant C57BL/6 mice. Following parasite injection, IL-27R-deficient mice (WSX.1 $^{-/-}$ mice) developed more severe lesions associated with the appearance of Th17 cells, showing that IL-27 is controlling the development of Th17 cells in *L. major* Sd susceptible mice (Anderson et al., 2009).

In contrast to the above studies, IL-17 was shown to correlate with protection against infection in C57BL/6 mice vaccinated with live *L. major* Friedlin and CpG (Wu et al., 2010). First, vaccination enhanced the development of Th17 cells, and second, vaccinated IL-17R $^{-/-}$ mice developed higher lesion sizes and parasite burdens at early but not later time points after infection. Despite the induction of an inflammatory response, no IL-23 was detectable at the site of parasite inoculation or in the dLN of vaccinated mice. These data suggest that the type of Th17 cells measured in this study resemble classical Th17 cells (Th17 β), which is different from those measured in the other studies that appear more similar to inflammatory Th17(23) cells (Lopez Kostka et al., 2009), although definitive confirmation of the existence of these Th17 subpopulations remains to be established in these experimental models. Similar vaccinations in BALB/c mice did not induce the development of Th17 cells (Wu et al., 2010).

Collectively these studies show that IL-17, whether produced by Th17 cells or another cell type, is a modulator of *L. major* infection. Furthermore, the impact of Th17 cells appears to differ significantly in response to distinct strains of *L. major* parasites and lesion development also varies depending strain, and thus the type of immune response.

THE DEVELOPMENT OF T_{reg} CELLS FOLLOWING L. MAJOR INOCULATION

CD4 $^{+}$ Foxp3 $^{+}$ T_{reg} cell functions involve the maintenance of immune tolerance, as well as the prevention of inflammatory diseases. In addition, these cells are critical in regulating the T helper immune response generated in response to infection, reviewed in Campbell and Koch (2011). The balance of various cytokines including IL-6, IL-1, IL-23, and retinoic acid links the development of T_{regs} to that of Th17 cells (Lochner et al., 2008; Yang et al., 2008; Zhou, 2008).

The role of CD4 $^{+}$ Foxp3 $^{+}$ T_{regs} in the development of Th1 cells during *L. major* infection was elegantly demonstrated by Belkaid and colleagues. They first described the accumulation of T_{regs} at the site of parasite inoculation (skin) in C57BL/6 mice. The presence of T_{regs} suppressed the ability of effector Th1 cells to totally eliminate *L. major*, allowing the maintenance of concomitant immunity after clinical cure (Belkaid et al., 2002). Depletion of T_{regs} in C57BL/6 mice enhanced the development of Th1 cells, rendering *L. major* resistant strains of mice more resistant to the primary infection (based on smaller lesion size and increased Th1 cytokine production, and decreased parasite load) but susceptible to a challenge secondary infection (Belkaid et al., 2002). It was

further demonstrated that the IL-10 secreted by CD4⁺Foxp3⁺ T_{regs} was critical in preventing total elimination of the parasites in the skin (Suffia et al., 2005).

Unlike reports in C57BL/6 mice, the susceptibility to *L. major* in BALB/c IL-4Rα^{-/-} mice was not caused by the secretion of IL-10 by T_{regs} (Nagase et al., 2007). Furthermore, inoculation of C57BL/6 mice with the *L. major* Sd substrain showed that the susceptibility is dependent on the secretion of IL-10 by IFNγ-secreting CD4⁺ T cells and not by T_{regs} (Anderson et al., 2005, 2009).

We and others have shown that CD4⁺Foxp3⁺ T_{regs} also modulate the extent of *L. major*-specific Th2 immune responses. T_{regs} controlled the magnitude of infection in *L. major*-infected BALB/c mice. Depletion of T_{reg} cells prior to parasite inoculation enhanced parasite loads, Th2 responses, and lesion size rendering susceptible mice more susceptible to infection (Aseffa et al., 2002). Experimental reconstitution of SCID mice with T cells was further used to investigate the suppressive properties of T_{reg} cells. Transfer of 10⁷ naïve BALB/c spleen cells prior to infection led to resistance to *L. major*, associated with the development of a Th1 response, while the transfer of 10⁸ cells rendered the mice highly susceptible to infection, associated with the development of a Th2 immune response (Mitchell et al., 1981). Transfer of 10⁷ cells depleted of CD4⁺Foxp3⁺ T_{regs} suppressed the development of a protective Th1 response in reconstituted SCID mice infected with *L. major* (Aseffa et al., 2002; Liu et al., 2003; Xu et al., 2003), allowing the progressive development of an unhealing lesion linked to a Th2 response. The effect of CD4⁺Foxp3⁺ T_{regs} on the outcome of disease in these reconstituted SCID mice varied with the timing of CD4⁺Foxp3⁺ T_{reg} reconstitution with a major effect on the inflammatory lesion appearing late in infection (Aseffa et al., 2002; Liu et al., 2003; Xu et al., 2003). Altogether, the suppressive activity of CD4⁺Foxp3⁺ T_{regs} modulates both Th1 and Th2 *L. major*-driven immune responses. However, the relative importance of the IL-10 secreted by T_{regs} appears to differ in mice susceptible or resistant to infection, with a critical role in resistance to infection, but no suppressive role in unhealing lesions. A role for other types of regulatory T cells such as inducible T_{regs} in the control of *L. major* infection remains to be determined.

CONCLUSION

The Th1/Th2 paradigm is still considered as the basis of vaccine development and is used in pre-clinical trials. Indeed antigens that induce a Th1 response are considered as potential protective antigens and those inducing a Th2 response are associated with

pathology. Based on the observations (1) that the ablation of Th2 cytokines such as IL-4, IL-13, and IL-10 also confers resistance to infection and (2) that antigens such as LACK or cysteine protease antigens that strongly induce Th2 responses early after infection with *Leishmania* are protective when administered with appropriate adjuvants (Mougneau et al., 1995; Pollock et al., 2003); it appears that in addition to promoting a Th1 response, vaccination should also abolish the Th2 response induced by infection with *Leishmania* parasites. Additional work will be needed to understand if plasticity of the different T helper subsets occurs during *Leishmania* infection, before extrapolating results obtained in experimental cutaneous leishmaniasis for the design of new immuno-intervention tools or prophylactic approaches. Such work will also be important in defining surrogate markers of protection that may be used for diagnostics. IFNγ has been widely used as a marker of protection, however, as discussed here and shown in a recent study (Nylen et al., 2006), it may not always be the best marker for resistance.

The experimental model of *L. major* infection has and will continue to provide invaluable information on the mechanisms involved in T helper differentiation, with applications in leishmaniasis and other diseases where T helper subsets are contributing to pathology. It is becoming increasingly apparent that different strains of parasites causing cutaneous lesions, from the Old and New World, induce distinct types of immune responses with non-healing lesions often associated with the failure to develop a Th1 response rather than the induction of a CD4⁺ Th2 response, reviewed in Alexander and Bryson (2005). In order to apply the informations gained by studying the mechanisms of protection analyzed in experimental murine cutaneous leishmaniasis to humans, one should be careful to also take into account the diversity of immune responses induced by different substrains of parasites, as seen for *L. major*, as well as by other *Leishmania* species and strains causing cutaneous lesions in different parts of the world. Sequencing of the different substrains should provide important clues on the understanding of the parasite specific features leading to distinct immune responses.

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